US ARMY MEDICAL RESEARCH INSTITUTE OF CHEMICAL DEFENSE
ABERDEEN PROVING GROUND, MARYLAND 21010-5425

USAMRICD-TR-89-03

DIAZEPAM PHARMACOKINETICS IN RHESUS MACAQUE (MACACA MULATTA)

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May 1989

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UNCLASSIFIED
SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S) USAMRICD-TR-89-03	5. MONITORING ORGANIZATION REPORT NUMBER(S) USAMRICD-TR-89-03
6a. NAME OF PERFORMING ORGANIZATION US Army Med Rsch Inst of Chem Def	6b. OFFICE SYMBOL (If applicable) SGRD-UV-PA
7a. NAME OF MONITORING ORGANIZATION US Army Med Rsch Inst of Chem Def	7b. OFFICE SYMBOL (If applicable) SGRD-UV-RC
6c. ADDRESS (City, State, and ZIP Code) Aberdeen Proving Ground, Maryland 21010-5425	7b. ADDRESS (City, State, and ZIP Code) Aberdeen Proving Ground, Maryland 21010-5425
8a. NAME OF FUNDING/SPONSORING ORGANIZATION	8b. OFFICE SYMBOL (If applicable)
8c. ADDRESS (City, State, and ZIP Code)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
623002	3M263002	995 AA	

11. TITLE (Include Security Classification)

Diazepam Pharmacokinetics in Rhesus Macaque (Macaca Mulatta). (U)

12. PERSONAL AUTHOR(S)
LUKEY, B.J., CORCORAN, K.D., MCCLUSKEY, M.P., CLARK, C.R.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final Report	FROM Jun 88 TO Nov 88	May 1989	52

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)			
FIELD	GROUP	SUB-GROUP	Diazepam	Monkey	Intramuscular	Laboratory
06	15		Rhesus	Pharmacokinetic	RA V	animal

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The purpose of this study was (1) to define the maximum serum concentration of unbound diazepam for monkeys intramuscularly administered 100 ug diazepam/kg and (2) to estimate from the literature the dose that would produce the same maximum serum concentration of unbound diazepam in man.

Six rhesus monkeys were intramuscularly injected with 100 (ug) (micrograms) diazepam/kg in the hind limb. Blood (3 ml) was collected via an indwelling saphenous catheter immediately prior to and 5, 10, 15, 25, 40, 60, 90, 120, 180, and 240 minutes after diazepam dosing. A contract laboratory, blind of the labelling code analyzed diazepam serum concentrations by electron-capture gas chromatography and the percentage for unbound diazepam by equilibrium dialysis.

The concentration-time data for total (unbound and bound) diazepam fit a one-compartment open (open) model with first order absorption and elimination (C(t)=17.3

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	UNCLASSIFIED

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Richard P. Solana	(301) 671-2455	SGRD-UV-P

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE
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Block 19. Con't.

$(e^{-0.015t} - e^{-0.071t})$. Maximum serum concentration was 50 ng/ml. The percentage of diazepam unbound to serum proteins was 4.6% and therefore the maximum concentration of free diazepam was 2.3 ng/ml.

Eleven open literature studies that intramuscularly administered diazepam to adult, nonpregnant humans were used to correlate administered dose to maximum serum/plasma concentration of total diazepam. Numerous uncontrolled variables complicated the pooling of data from the literature. Nevertheless, least squares regression analysis of maximum concentration versus dose weighted by the sample size and force through 0,0 yielded the best fit line, y=14x. Because 1.3% of diazepam is free in human plasma, the same free concentration in man (2.3 ng/ml) requires 177 ng/ml total (free and bound) plasma concentration. The intramuscular dose estimate from the literature to produce 177 ng/ml in man is 12.4 mg diazepam. Due to variability in the literature, the intramuscular dose for man could range from 7.1 to 20 mg diazepam. (KT)

PREFACE

The work described in this report is authorized under USAMRICD animal use protocol number 1-02-88-000-A-493, entitled "DIAZEPAM PHARMACOKINETICS IN RHESUS MACAQUE (MACACA MULATTA)." The work was started on 6 Jun 88 and completed on 1 Nov 88. The experimental data are recorded in USAMRICD notebook number 042-88.

ACKNOWLEDGEMENTS

We express our appreciation for the superb technical support SSG Dennis Davis, SGT Brian Ling, SGT James Beerman and PFC David Gillis gave in handling and preparing the animals. We thank SP4 Scott Heykamp for the determination of hematocrits and serum protein. Gratitude is also extended to Dr. Tsung-Ming Shih for assistance in the literature search. Special thanks are extended to Mrs. Marita Lukey and Mrs. Del. Corcoran for supporting the after hours work on this project. Also, we thank our contractor, Dr. David J. Greenblatt, who provided measurements of desmethyldiazepam concentrations and the free (unbound) diazepam concentrations without charge.

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INTRODUCTION

Soman-induced convulsions have been shown to increase the incidence of brain lesions in nonhuman primates (Wall et al., 1987) and, therefore, may very likely cause permanent brain damage in man. The U.S. Army desires to prevent these convulsions, thereby increasing the soldier's chance of survival during a chemical warfare attack as well as the quality of life afterwards. Currently diazepam is being investigated as an anticonvulsant treatment, concomitantly administered with atropine and 2-pralidoxime chloride for organophosphate poisoned soldiers.

The minimum effective intramuscular dose of diazepam, 100 ug/kg, was determined in nonhuman primates pretreated with pyridostigmine, poisoned with soman and treated with atropine, 2-pralidoxime and diazepam (Hayward, 1988). Because efficacy studies are not performed in man, the minimum effective dose for man will be extrapolated from primate studies.

Extrapolation to obtain the same therapeutic response is most accurately achieved by equating drug concentration at the active site. The anticonvulsant effect of diazepam is centrally mediated, and therefore, measurement of diazepam concentrations at the receptor site (in the brain) is not feasible. However, diazepam concentrations in the cerebral spinal fluid (CSF) are expected to relate to its concentration at the active receptor and therefore also relate to the therapeutic response. Unfortunately, CSF is difficult to obtain, especially when serially sampling for pharmacokinetic studies.

Several studies have found that the total concentration of diazepam in the CSF correlates highly with unbound diazepam concentrations in plasma (Greenblatt et al., 1980; Arendt et al., 1983; Kanto et al., 1975). Plasma samples can be easily obtained and total diazepam concentration (plasma bound and free) can be easily analyzed. Because very little diazepam is not bound to plasma protein (1.3% for young males; Greenblatt et al., 1980a), measurement of free concentrations requires a very sensitive method, especially at the early and late phases of a free concentration-time profile. Protein binding is not concentration dependent (Moshitto and Greenblatt, 1983), and therefore disposition of total drug should parallel free. The percentage of diazepam free could be determined at a high concentration, and that percentage should remain constant throughout the study. The free concentration-time profile could therefore be determined from total concentrations.

The extrapolation of the diazepam dose from monkey to man requires defining diazepam pharmacokinetic parameters and percent of diazepam free in plasma for both man and monkey. Pharmacokinetic parameters have been reported in the literature for man (Table I) but not for monkey.

This study determines the peak serum concentration of diazepam achieved in the nonhuman primate when given the minimum effective intramuscular dose for controlling soman-induced seizures. Also this study determines the percent of diazepam not bound to plasma protein in the monkey. This information in conjunction with the human literature is used to extrapolate the required

human intramuscular dose necessary to achieve comparable free diazepam levels in serum of man.

MATERIALS AND METHODS

A. Experimental Design

Six male rhesus primates were issued from the USAMRICD nonhuman primate colony IAW SGRD-UV-VM Memorandum 40-15 "Macaque Husbandry and Quarantine Procedures," July 1985.

The study was conducted in room 237, building 3081. All personnel handling the nonhuman primates or working within the room wore a surgical mask, a lab coat, and latex protective gloves.

Each animal was placed in a plexiglass restraint chair for training in order to acclimate the animal to the apparatus. Four training sessions (1-3 hours each) were conducted for every animal prior to the day of the experiment. During the training sessions the nonhuman primates were offered fruit as a positive reinforcement. Training was under supervision of MAJ Kevin Corcoran, the Veterinarian in Charge of the nonhuman primate colony.

Animals were fasted overnight prior to the day of experimentation. Two different animals were dosed each day for three consecutive days. Prior to the day of dosing, the monkey's legs were clipped from the pelvis to the tarsus. Animals were placed in plexiglass restraint chairs for blood sampling and remained in the restraint chairs for no more than six hours.

A 20 gauge 2 inch percutaneous teflon catheter[1] was introduced into a saphenous vein and flushed with 0.9% saline.[2] Blood samples were taken through a four-way stopcock[3] connected to the catheter by an eight inch plastic tubing extension. The extension was fabricated from eight inches of the male end of a 30 inch extension set[4] connected to a female size 3 stainless steel plastic tubing adapter.[5] The total volume of the catheter plus the extension was 1.4 ml. Between blood samples the catheter and extension were continuously flushed with 0.9% normal saline at a rate of 3 ml/hr via a continuous flush device.[6] Saline was delivered under 300 mg Hg

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[1]Angiocath[R] No. 2818, The Deseret Company, Sandy, UT 84070

[2]Sodium Chloride injection USP, Travenol Laboratories, Inc., Deerfield, IL 60015

[3]Pharmaseal[R] No. K72, Pharmaseal Inc., Toa Alta, PR 00758

[4]No. 4610, Abbott Hospitals, Inc., North Chicago, IL 60064

[5]No. 7543, Becton, Dickinson and Company, Parsippany, NJ 07054

[6]INTRAFLOW[R] II No. 42002-01, Sorenson Research, Salt Lake City, UT 84123

pressure to the flush device via a pediatric intravenous injection set[7] attached to a pressure bag.[8]

Diazepam (100 ug/kg) was administered to each nonhuman primate in the large muscle mass at the rear of the hind limb (opposite the leg catheterized) between the hip and knee. The dosing solution (5 mg/ml) was delivered via a 250 ul Hamilton syringe (#725) and a 23 gauge 1.25-inch needle. The injection was given as a bolus dose deep into the muscle. The plunger of the syringe was withdrawn slightly before expulsion of the dose to insure that the needle was in the muscle and not a blood vessel. If no blood came back into the syringe, the needle was assumed to be in the muscle.

Sampling times had been chosen from a pilot study (Lukey, 1988) to determine the maximum diazepam serum concentration. These sampling times were prior to and 5, 10, 15, 25, 40, 60, 90, 120, 180 and 240 minutes after injection. Before removing each blood sample, a 2 ml presample was taken to remove saline from the catheter. Blood volume for each sample was 3 ml. Following each collection, 5 ml of 0.9% normal saline was injected through the catheter to replace the fluid volume removed. Between samples the catheter was attached to the continuous flush system. For each blood sample, the animal lost approximately 3.5 ml of blood. A maximum of eleven blood samples were taken from each monkey, resulting in a total blood loss of approximately 38.5 ml.

Microhematocrit and plasma total protein determinations were performed on the 0-, 40-, 90- and 240-minute blood samples to assure anemia had not developed. Two disposable heparinized soda lime glass microhematocrit capillary tubes[9] were filled with blood and one end of each tube was plugged with clay. The capillary tubes were centrifuged at 10,000 rpm for five minutes.[10] The hematocrit was determined using a microcapillary reader.[11] The capillary tubes were then scribed and broken to load the plasma into a refractometer[12] to determine the plasma total protein. The hematocrit and plasma protein determinations from both capillary tubes taken at each time point were averaged.

Two additional microhematocrit and plasma total protein determinations were performed at 24 hours and seven days following diazepam injection via venipuncture in order to document further changes in these parameters.

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[7]FLU VEN^{R} No. 0161-A, VENUSA, LTD., New York, NY 10017

[8]Sorenson Research Corp., Salt Lake City, UT 84123

[9]No. 73810, Kimble Corp., Toledo, OH 43666

[10]Model MB, Damon/IEC, Needham, MA 02194

[11]No. 2201, International Equipment Co., Boston, MA

[12]Model 10406, American Optical Corp., Buffalo, NY 14125

Blood samples were drawn into sterile disposable 5 ml plastic syringes and then placed into new generic glass disposable test tubes (10 mm x 75 mm) devoid of anticoagulant and sealed with parafilm. Samples were placed on ice until collection was completed and then refrigerated overnight to allow the clot to form. The following day the serum was transferred via pasteur pipette into new generic plastic disposable test tubes (10 mm x 75 mm) and centrifuged at 2500 rpm for 10 minutes. Serum was again transferred via pasteur pipette into new generic plastic screwtop tubes (10 mm x 75 mm) and frozen at -70°C. After the final sample was obtained, the catheter was removed and the animal returned to the care of the Veterinary Medicine and Surgery Branch. At the end of the week, samples were packaged in dry ice and sent by overnight delivery to David J. Greenblatt, M.D., Room 602, M-V Building, Tufts University School of Medicine, 136 Harrison Avenue, Boston, Massachusetts, 02111. Procedures for handling samples for diazepam analysis are found in Appendix A.

Serum samples were analyzed using electron-capture gas chromatography (Contract purchase # DAAL05-88-M-N384; Greenblatt et al., 1980b). The internal standard for this assay was an iodinated benzodiazepine analogue (Ro7-9749). Serum samples were extracted with benzene (containing 1.5% isoamyl alcohol). The organic phase was evaporated to dryness and the sample reconstituted with toluene (containing 15% isoamyl alcohol).

The chromatograph was equipped with 4 ft x 2 mm glass column containing 3% SP-2250 on 80/100 Supelcoport and operated at 250°C. The carrier gas was argon:methane (95:5) with a flow rate of 25 ml/min.

Accuracy and precision of analytical results were defined prior to the pharmacokinetic study as follows. Blank monkey serum was spiked with known amounts of the diazepam solution (5 ug/ml) by serial dilution to achieve the following concentrations: 0, 6.25, 12.5, 25, 50, 100 and 200 ug/ml serum. Each concentration was divided into three 1.0 ml aliquots. All test tubes were coded with random numbers, frozen at -70°C and transported to Dr. Greenblatt the same day. Precision was expressed as the coefficient of variation of the three samples for each concentration. Accuracy was the deviation of reported concentrations from expected.

For additional assurance of analytical accuracy and precision, six samples for each of two concentrations (50 and 100 ng/ml) were coded with random numbers and sent with samples obtained from the pharmacokinetic study in the rhesus monkey. Accuracy and precision of these controls were determined as above.

Protein binding of diazepam in serum was determined by equilibrium dialysis according to the method of Woo and Greenblatt (1979).

B. Data analysis

Plasma concentration-time data for each animal were fit to standard pharmacokinetic models using the PCNONLIN computer program (Statistical Consultants Inc., 1986). Initial estimates for each nonhuman primate were determined from the computer program JANA (Statistical Consultants, Lexington, KY). Data for model fitting were iteratively reweighted by the squared

reciprocal of the predicted concentration. Determination of the appropriate model was based upon a minimal sum of squared residuals, large correlation coefficient, small standard deviations of parameter estimates and unbiased distribution patterns of residuals of estimates of observed versus predicted values.

Maximum serum/plasma diazepam concentrations were obtained from the literature for adult, nonpregnant humans intramuscularly administered diazepam (Valium®). Weighted linear regression analysis was employed to define the best fit line for maximum concentration as a function of the diazepam dose. Weight was based upon the sample size except for the 0,0 point, which received a weight of 1000 to force the line through the origin.

RESULTS

Reported concentrations of quality control samples for defining the accuracy and precision of the analytical technique prior to the pharmacokinetic study are recorded in Appendix B. A contaminant was found in the monkey plasma which partially coeluted with diazepam. The contaminant caused blank samples to be reported with an average concentration of 20.7 ng/ml. Assuming equal interferences in all samples, the reported concentration for the blank (20.7 ng/ml) was subtracted from reported concentrations of other samples. Coefficients of variations for the corrected concentrations ranged from 2 to 20% and averaged 10%. Accuracy reported as % bias ranged from -15% to 8% and averaged -4%.

Quality control samples analyzed during the pharmacokinetic portion of this study did not have a contaminant interfering with diazepam quantification. The concentrations are listed in Table II. Coefficients of variation for 50 and 100 ng/ml were 8% and 5% respectively. Bias for 50 and 100 ng/ml were 5% and 2% respectively.

The hematocrit and total plasma protein were observed in the animals for 24 hours after diazepam dosing. The average hematocrit slightly decreased from 42% at time 0 to 35% at time 24 hrs (Table III). Plasma protein content decreased from 7.6 g/dl at time 0 to 6.5 g/dl at 4 hrs but returned to normal at 24 hrs (Table IV). No overt signs or symptoms of anemia were noted. Animal activity after diazepam dosing appeared no different than during the last phases of chair restraint training. No animals slept or even appeared drowsy after dosing.

Diazepam and metabolite (desmethyldiazepam) serum concentrations are tabulated for all animals with respect to time (Table V) Diazepam concentration-time data for each animal fit a one-compartment, open model with first order absorption and elimination (Appendix C). Desmethyldiazepam did not fit the model well because the elimination phase was not observed. Nevertheless, the model was employed to get a rough estimate of metabolite appearance (Appendix D).

Pharmacokinetic estimates for each animal are reported in Appendix E and the average extimates are listed in Table VI. From the averages of the

pharmacokinetic estimates, diazepam and desmethyldiazepam serum concentration-time profiles were obtained. In order to represent raw data on Figure 1, concentrations among animals were averaged with respect to time (Table VII), and the resulting means and S.E.M. were displayed. From the diazepam best fit line, the maximum diazepam serum concentration averaged 50 ng/ml at 29 minutes (Table VI).

The percentage of diazepam unbound to serum protein averaged 4.6 ± 0.7% (Table VIII). Therefore, the maximum serum concentration of unbound diazepam is 2.3 ng/ml.

DISCUSSION

The first set of quality control samples were used to assure that Dr. Greenblatt's laboratory could accurately report diazepam concentrations within the desired range before the pharmacokinetic study would begin. Because he was blind to the expected concentrations, Dr. Greenblatt did not know which samples were blanks. However, he noted a contaminant partially coeluted with diazepam and informed us that the reported concentration would be consistently higher than expected. The blank samples revealed that the contaminant artificially increased the reported diazepam concentration by 21 ng/ml. When the value was subtracted from reported concentrations, the results were very close to expected. We therefore chose this contractor since his was the only available laboratory that could accurately measure diazepam concentrations within our desired concentration range and our time limitations.

Quality control samples were also sent with the pharmacokinetic samples to assure accurate and precise determination of diazepam concentration during the study. Fortunately, no contaminant appeared in any animal's serum and therefore no adjustments needed to be made. Also Dr. Greenblatt's analysis provided quantification of the desmethyl diazepam metabolite with no additional cost.

Diazepam serum concentration-time profile of each animal fit the one compartment open model well. Human data obtained from intravenous administration of diazepam indicate the concentration-time curve is best described by a two-compartment model with distribution and elimination half lives approximating 1 and 32 hrs respectively (Klotz et al., 1980). Since our study was not conducted past 4 hrs, the elimination phase may appear to have been not detectable, and therefore, the apparent decrease in diazepam concentration would primarily have been due to the distribution of the compound. However, analysis of the diazepam metabolite fate indicates otherwise.

Although the study was not designed to define the pharmacokinetic profile of the desmethyl diazepam, estimates were made. The elimination phase was not clearly apparent from the concentration-time profile, and therefore estimates of this phase are weak. However the formation phase was adequately defined by fitting data to a one-compartment open model with first order absorption, first order elimination and a lag time. The diazepam metabolite formation parallels diazepam disappearance and metabolite concentrations approach those

of diazepam, indicating that the major route of diazepam elimination was its metabolism to desmethyldiazepam. The data indicate that our diazepam model truly defines diazepam elimination, rather than distribution expected from human studies (Klotz et al., 1980).

From the diazepam model, an average estimate of the maximum diazepam concentration was 50 ng/ml at 29 min from a 0.1 mg/kg intramuscular dose. The maximum diazepam serum concentration in the pilot study (Lukey, 1988) was 128 ng/ml for a single rhesus macaque intramuscularly administered 0.2 mg/kg. When the maximum serum concentration versus dose for the pilot study and protocol were graphed, a least squares regression line for the two points and the origin resulted in a correlation coefficient (r) of 0.992. Because the study was not designed to define that relationship, the excellent correlation must not be weighted too heavily. Linearity suggests the pharmacokinetics behave linearly in the dose range studied and indicates reproducibility in the study.

The maximum serum concentration of unbound diazepam was estimated as 2.3 ng/ml. We estimated from the literature man's intramuscular diazepam dose that produces the same maximum serum concentration of free diazepam found in monkeys receiving 0.1 mg/kg (2.3 ng/ml). Numerous studies have investigated the clinical pharmacokinetics of diazepam in man, but few have studied the pharmacokinetics by the intramuscular route of administration. No single study reported the relationship between maximum diazepam concentrations in serum and intramuscularly administered doses. Several studies that meet the criteria (adult, nonpregnant humans receiving i.m. diazepam) were pooled to define this relationship. These experiments were conducted in different laboratories using different analytical techniques and at different times. Injection sites were not standardized. Needle lengths were not always reported and those that were reported varied. Some studies involved men, some involved women and others did not specify. The numerous variables complicate pooling of the literature studies.

Bearing in mind the complications, we found the maximum serum concentrations for man linearly related to the diazepam i.m. dose (C_{max} (ng/ml) = 14.3*dose(mg)). A graphic representation of the relationship is depicted in Figure 2, and specifics of the data are reported in Table I. The relationship is for total diazepam concentration (bound and free). Since 1.3% of diazepam is unbound in serum (Greenblatt et al., 1980a), the desired 2.3 ng/ml free concentration corresponds to a total concentration of 177 ng/ml. From the above relationship (Figure 2), the estimated human i.m. dose expected to produce a 177 ng/ml maximum concentration is 12.4 mg. Figure 3 displays the extreme upper and lower doses which produce the C_{max} from the two regression lines that beginning at the origin and encompass all data. The resulting doses are 7.1 and 20 mg diazepam.

Only one study that intramuscularly administered diazepam to humans defined the pharmacokinetics adequately for graphic comparison of the concentration-time profiles with those of monkeys (Wichlinski et al., 1985). The study administered 10 mg to 12 subjects and determined the best fit curve for the average concentration-time profile. For comparison with our data, the curve needed to be modified. By using the reported average weight of 75 kg and

assuming pharmacokinetic linearity, the best fit curve was extrapolated from human study to represent a concentration-time profile for a 0.1 mg/kg dose. In Figure 4, the much more elimination of diazepam in the monkey is apparent.

CONCLUSION

The maximum serum concentration of diazepam for monkeys intramuscularly administered 100 ug diazepam/kg was 50 ng/ml. The percentage of diazepam unbound in serum was 4.6%. Therefore the maximum concentration of unbound diazepam was 2.3 ng/ml. Because 1.3% of diazepam is free in human plasma, the same free concentration in man (2.3 ng/ml) requires 177 ng/ml total (free and bound) plasma concentration. The intramuscular dose estimate from the literature to produce 177 ng/ml in man is 12.4 mg diazepam. Due to variability in the literature, the intramuscular dose for man could range from 7.1 to 19.7 mg.

Assuming that man and monkey respond equally to the same maximum serum concentration of unbound diazepam, this human dose is the predicted minimal effective dose to inhibit soman-induced convulsion with the current pretreatment/treatment therapy. The dose, based upon numerous uncontrolled variables from the human literature and this limited pharmacokinetic study in the rhesus macaque, is a rough estimate that approximates man's minimal dose.

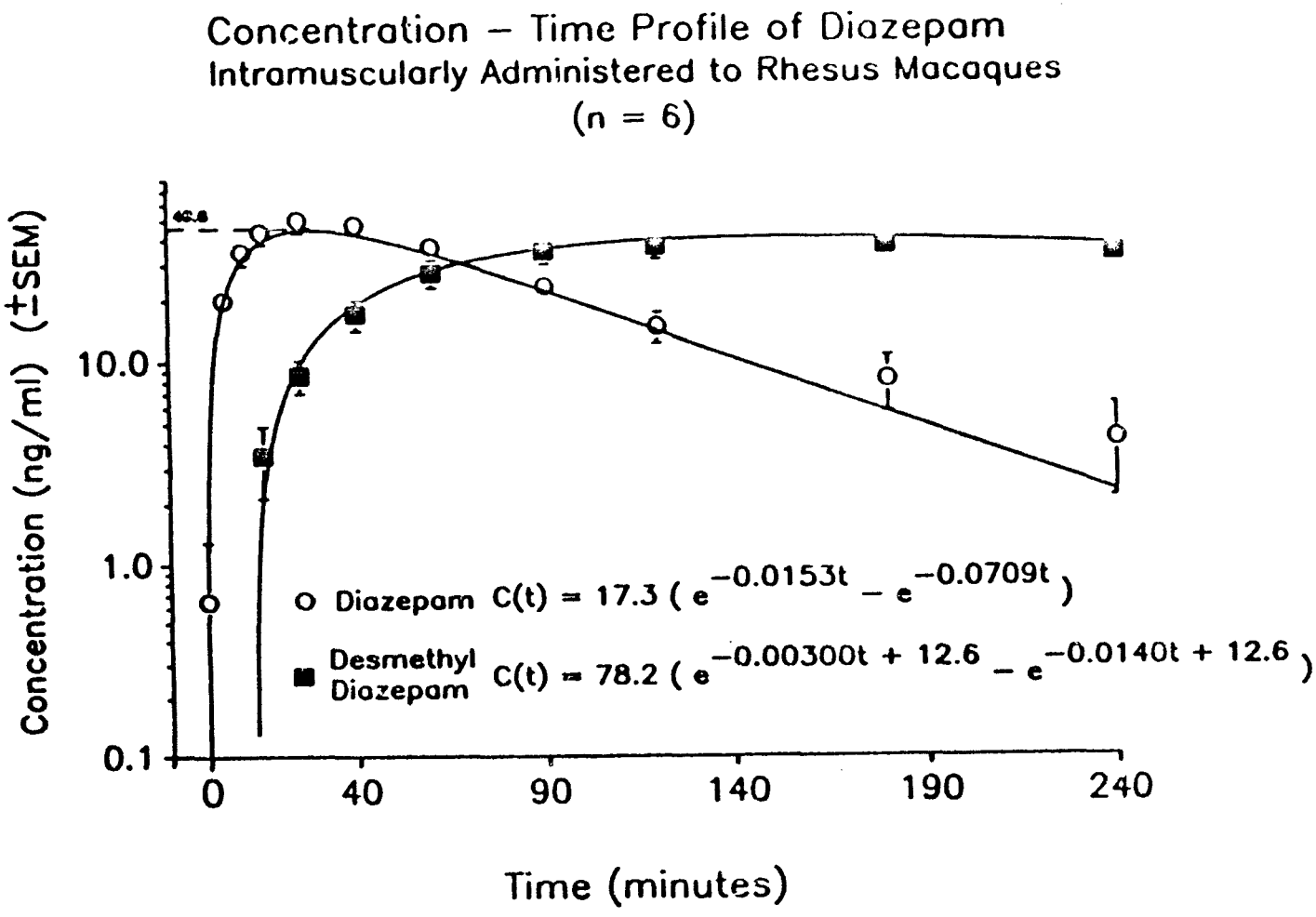

Figure 1. Diazepam and desmethyldiazepam serum concentration-time relationships in the rhesus monkey after an intramuscular administration of diazepam fit a one compartment open model with first order absorption and elimination. The best fit line was determined from average pharmacokinetic estimates for each animal (n=6). Mean concentrations were not used to estimate pharmacokinetic estimates but are displayed with S.E.M. to represent raw data.

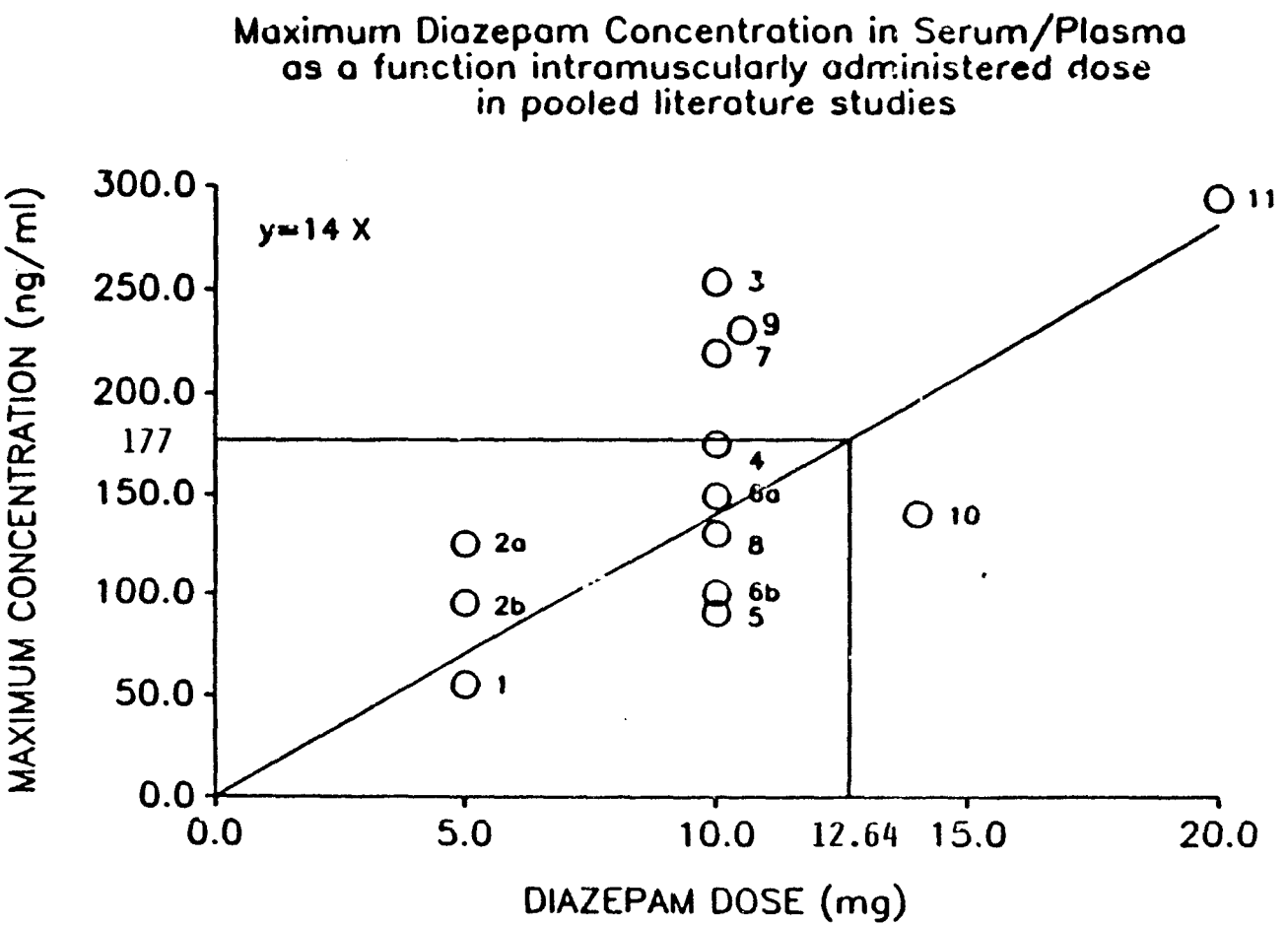

Figure 2. The maximum diazepam concentration in serum, plasma was plotted as a function of intramuscularly administered dose in pooled literature studies. Each data point represents a group of individuals within a study. Labels for data points are key to Table I for specific information about that group. The least squares regression line was weighted by sample size and was force through the origin.

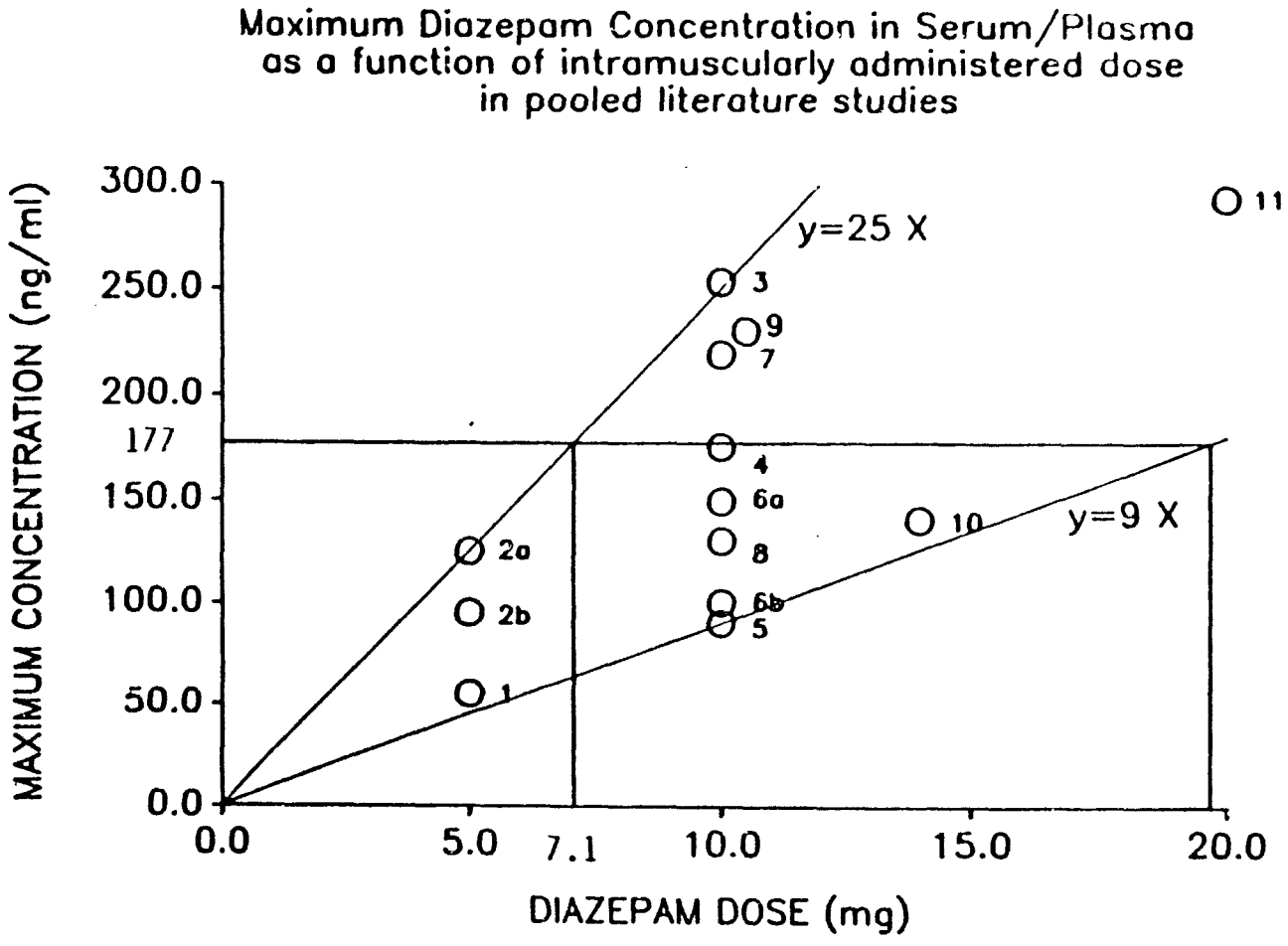

Figure 3. The maximum diazepam concentration in serum/plasma was plotted as a function of intramuscularly administered dose in pooled literature studies. Each data point represents a group of individuals within a study. Labels for data points are key to Table I for specific information about that group. The upper and lower limits are lines originating at the origin and incorporating all data points within their boundaries.

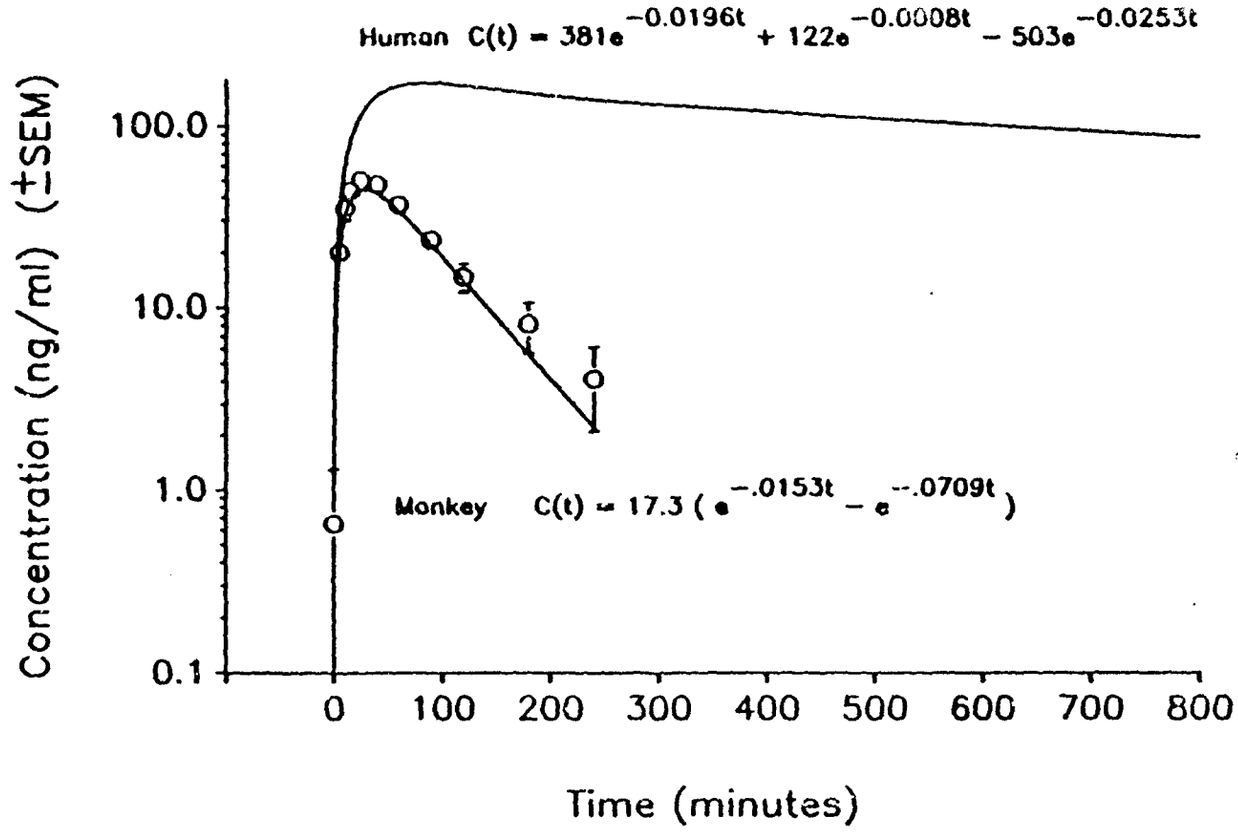

Figure 4. Diazepam concentration-time profile resulting from an intramuscular administration was compared in rhesus monkey and man. The curve representing human data was obtained from the best fit line in a study which dosed subjects (n= 12) with 10 mg diazepam intramuscularly. By using the reported average weight of 75 kg and assuming pharmacokinetic linearity, the illustrated line above was extrapolated from human study to represent a concentration-time profile for a 0.1 mg/kg dose. The curve for monkey was obtained from 6 animals intramuscularly 0.1 mg/kg diazepam.

TABLE I. Literature review of studies intramuscularly dosing diazepam (Valium^{R}) to adult, nonpregnant humans and observing maximum serum/plasma concentrations.

Code Fig 1	Dose (mg)	Sample size	M	F	Site	T_{max} (min)	C_{max} (ng/ml)	Reference
1	5	10	5	5	?	120	55	Kanto, 1974
2a	5	5	5	0	deltoid	33	125	Divoll et al., 1983
2b	5	5	0	5	deltoid	360	95	Divoll et al., 1983
3	10	4	?	?	?	<10	253	Baird et al., 1973
4	10	12	11	1	?	90	170	Wichlinski et al., 1985
5	10	9*	6	3	?	128	90	Thorn-Aquist et al., 1977
6a	10	33	0	33	thigh	90	149	Gamble et al., 1975
6b	10	10	0	10	buttock	90	100	Gamble et al., 1975
7	10	10	?	?	?	60	219	Resch et al., 1986
8	10	3	?	?	?	15	130	DeSilva et al., 1966
9	10.5	7	?	?	thigh	90	230	Kortilla et al., 1976
10	14**	18	?	?	thigh	45	140	VonDardel et al., 1983
11	20	6	?	?	?	60	293	Hillestad et al., 1979

? Information was not reported.

* 60% of the subjects in the report were males. Although all were not used to determine the above information, we assumed the same proportion existed in this sample group.

** The dose was reported as 0.2 mg/kg, corresponding to 14 mg for a 70 kg man.

TABLE II. Quality control samples analyzed concurrently with pharmacokinetic samples.

DIAZEPAM CONCENTRATION (ng/ml)		
Expected	Detected	% Bias
50	43.8	12.4
50	50.5	-1.0
50	51.2	-2.4
50	49.3	1.4
50	47.9	4.2
50	42.1	15.8
Mean	47.5	5.1
S.D.	3.7	7.4
C.V.	7.8	
100	92.4	7.6
100	101.7	-1.7
100	102.5	-2.5
100	99.7	0.3
100	100.5	-0.5
100	91.4	8.6
Mean	98.0	2.0
S.D.	4.9	4.9
C V.	5.0	

TABLE III. Hematocrit levels analyzed relative to diazepam dosing.

HEMATOCRIT (%)

ANIMAL ID	Sequence Number	HOURS 0	0.67	1.5	4	24	168
18386	1	40%	35%	39%	39%	31%	38%
13379	2	43%	41%	39%	33%	39%	40%
DA143	3	43%	40%	39%	39%	35%	40%
DA158	4	39%	35%	34%	30%	32%	35%
P-417	5	45%	44%	44%	40%	38%	43%
13432	6	42%	41%	40%	35%	33%	38%
AVERAGE		42%	39%	39%	36%	35%	39%

TABLE IV. Blood protein levels analyzed relative to diazepam dosing.

TOTAL PROTEIN (g/dl)

ANIMAL ID	Sequence Number	HOURS 0	0.67	1.5	4	24	168
18386	1	7.4	7.0	7.1	7.4	7.6	7.5
13379	2	8.0	7.2	7.2	5.5	7.8	8.0
DA143	3	7.7	7.2	6.7	6.7	7.4	8.2
DA158	4	7.4	6.6	6.4	6.1	7.5	8.2
P-417	5	7.5	7.3	7.4	7.0	7.5	8.5
13432	6	7.5	7.7	7.0	6.1	7.2	7.8
AVERAGE		7.6	7.2	7.0	6.5	7.5	8.0

TABLE V. Serum concentrations of diazepam and desmethyl diazepam in individual animals.

DIAZEPAM CONCENTRATION (NG/ML)

Time (min)	Animal Number 1	2	3	4	5	6
0	0.0	0.0	3.9	0.0	0.0	0.0
5	17.8	20.3	20.1	17.5	29.1	15.2
10	24.8	30.0	51.1	28.5	51.1	24.0
15	33.2	36.4	57.2	40.4	67.2	28.9
25	37.2	35.8	71.2	53.9	70.9	32.9
40	40.6	37.4	60.2	43.2	68.6	34.8
60	34.3	27.5	35.5	39.3	46.9	35.7
90	25.1	17.2	18.6	21.3	28.5	28.5
120	17.3	11.8	12.7	4.5	19.1	22.2
180	10.9	0.0	4.1	8.9	7.1	17.6
240	8.8	0.0	0.0	2.1	2.1	11.6

DESMETHYL DIAZEPAM CONCENTRATION (NG/ML)

Time (min)	Animal Number 1	2	3	4	5	6
0	0.0	0.0	0.0	0.0	0.0	0.0
5	0.0	0.0	0.0	0.0	0.0	0.0
10	0.0	0.0	0.0	0.0	0.0	0.0
15	0.0	7.2	7.4	2.4	4.1	0.0
25	7.8	9.2	13.6	6.9	11.5	2.4
40	13.7	20.2	25.2	15.1	22.5	5.2
60	25.1	29.2	36.7	24.3	37.6	8.8
90	30.6	41.3	43.4	35.9	44.4	12.4
120	34.4	43.0	46.2	32.3	47.5	15.1
180	42.4	38.8	36.4	37.5	46.5	18.2
240	38.7	41.3	33.6	27.7	37.7	21.3

TABLE VI. Mean pharmacokinetic parameters (S.D.) of diazepam and the desmethyl metabolite in the rhesus monkeys

		DIAZEPAM		DESMETHYL DIAZEPAM	
		Mean	S.D.	Mean	S.D.
Vd	L/kg	1.47	0.53	UNDEFINED	
k01	1/min	0.355	0.043	0.014	0.006
k10	1/min	0.077	0.031	0.003	0.001
AUC	ng*min/ml	5494	1348	20760	5328
Cl	ml/min/kg	19.4	5.2	UNDEFINED	
t1/2 of k01	(min)	9.9	1.2	72.2	59.7
t1/2 of k10	(min)	58.0	32.6	233.5	63.2
Tmax	min	28.7	3.4	169.8	57.6
Cmax	ng/ml	49.6	13.2	38.4	8.4
Tlag	(min)	0	0	12.6	1.6

TABLE VII. Cumulative raw data for diazepam intramuscularly administered to rhesus macaques (n = 6).

Time (min)	DIAZEPAM Conc (ng/ml)	S.E.M.	DESMETHYL DIAZEPAM Conc (ng/ml)	S.E.M.
0	0.65	0.65	0.00	0.00
5	20.00	1.98	0.00	0.00
10	34.92	5.20	0.00	0.00
15	43.88	6.13	3.52	1.36
25	50.32	7.21	8.57	1.59
40	47.47	5.58	16.98	2.95
60	36.53	2.60	26.95	4.29
90	23.20	2.00	34.67	4.93
120	14.60	2.57	36.42	4.96
180	8.10	2.46	36.63	3.98
240	4.10	2.00	33.38	3.10

TABLE VIII. Free fraction of diazepam in pooled primate sera

ANIMAL ID	SEQUENCE NUMBER	%UNBOUND
13386	1	5.0
13379	2	5.0
DA143	3	4.0
DA158	4	4.5
P-417	5	3.7
13432	6	5.4
AVERAGE		4.6
S.D.		0.7

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APPENDIX A

PROCEDURE FOR SERUM SAMPLE HANDLING

David J. Greenblatt, M.D.
Division of Clinical Pharmacology
Tufts-New England Medical Center

PROCEDURE FOR HANDLING OF SAMPLES FOR ANALYSIS OF BENZODIAZEPINES

Timing

Blood samples should be drawn prior to a maintenance dose for patients on multiple-dose regimens. The ideal sampling time for patients on daytime administration schedules is in the morning prior to the first dose of the day. For patients taking single bedtime doses of anxiolytics or hypnotics, the sample can be drawn in the morning, or in the evening prior to the dose. For subjects in single-dose pharmacokinetic studies, samples are drawn at whatever times following dosage are indicated in the protocol.

Tubes

Evacuated blood-collection tubes are the most convenient means of obtaining samples. Since plasma and serum yield nearly identical results, green-top (heparinized) tubes are the easiest to handle. However Venoject and Vacutainer brand tubes are not identical. Venoject tubes contain unknown contaminants that interfere with the analysis of diazepam, desmethyldiazepam, lorazepam, and triazolam. Therefore Venoject tubes should not be used. The recommended collection tube is: Vacutainer Brand (Becton-Dickinson Co, Rutherford, NJ 07070), catalogue number 6541, green-top and heparinized.

If the direct venipuncture technique is used, the full draw of the tubes is approximately 10 ml which is the proper sample size. If a syringe is used to obtain the blood, the syringe should be either all glass or disposable plastic form Becton Dickinson. Other disposable syringes (Monoject, Pharmaseal) contain contaminating substances and must not be used. Samples drawn by syringe are then transferred to a tube as described above.

Processing and Storage

Benzodiazepines are very stable in plasma -- samples once drawn need not be processed immediately or urgently. Whole blood samples (uncentrifuged) are stable at room temperature for 12 hours, and for 24 hours if refrigerated. However, do not freeze uncentrifuged samples.

After centrifugation, the upper plasma/serum layer is transferred to a plastic sample storage tube (Becton Dickinson is recommended). Red cells are discarded. The tube is capped and taped tightly closed (to avoid leakage in case it thaws during shipping). The tube is labeled with patient identification (name or number), date drawn, and time drawn. USE AN INDELIBILE MARKER. Water-based inks will wash off, rendering the sample uninterpretable.

Serum/plasma samples are frozen at -4°C or lower until the time of shipping. Frozen samples are stable indefinitely.

Shipping

Samples are packed in dry ice and shipped via Federal Express (priority one), or its equivalent, to Boston. The shipping day should be a Monday or Tuesday to avoid possible weekend delays. The shipping address is:

David J. Greenblatt, M.D.
Room 602, M-V Building
Tufts University School of Medicine
136 Harrison Avenue
Boston, MA 02111

Please note that this is only the shipping address.

All other mail should go to:

David J. Greenblatt, M.D.
Division of Clinical Pharmacology
Box 1007
Tufts-New England Medical Center
171 Harrison Avenue
Boston, MA 02111

Communication

Please call a day or so before shipping samples so Dr. Greenblatt can be looking for them. Also please call if there are any questions or if you must deviate from the above guidelines. If, for any reason, Dr. Greenblatt is not available, one of his colleagues (Ms. Ann Locniskar) will be able to help you.

Phone numbers: (617) 956-6997 (Division of Clinical Pharmacology)
(617) 332-4544 (Home number for nights and weekends)

APPENDIX B

QUALITY ASSURANCE OF ANALYTICAL METHOD PRIOR TO THE STUDY

QUALITY CONTROL FOR DIAZEPAM MONKEY STUDY

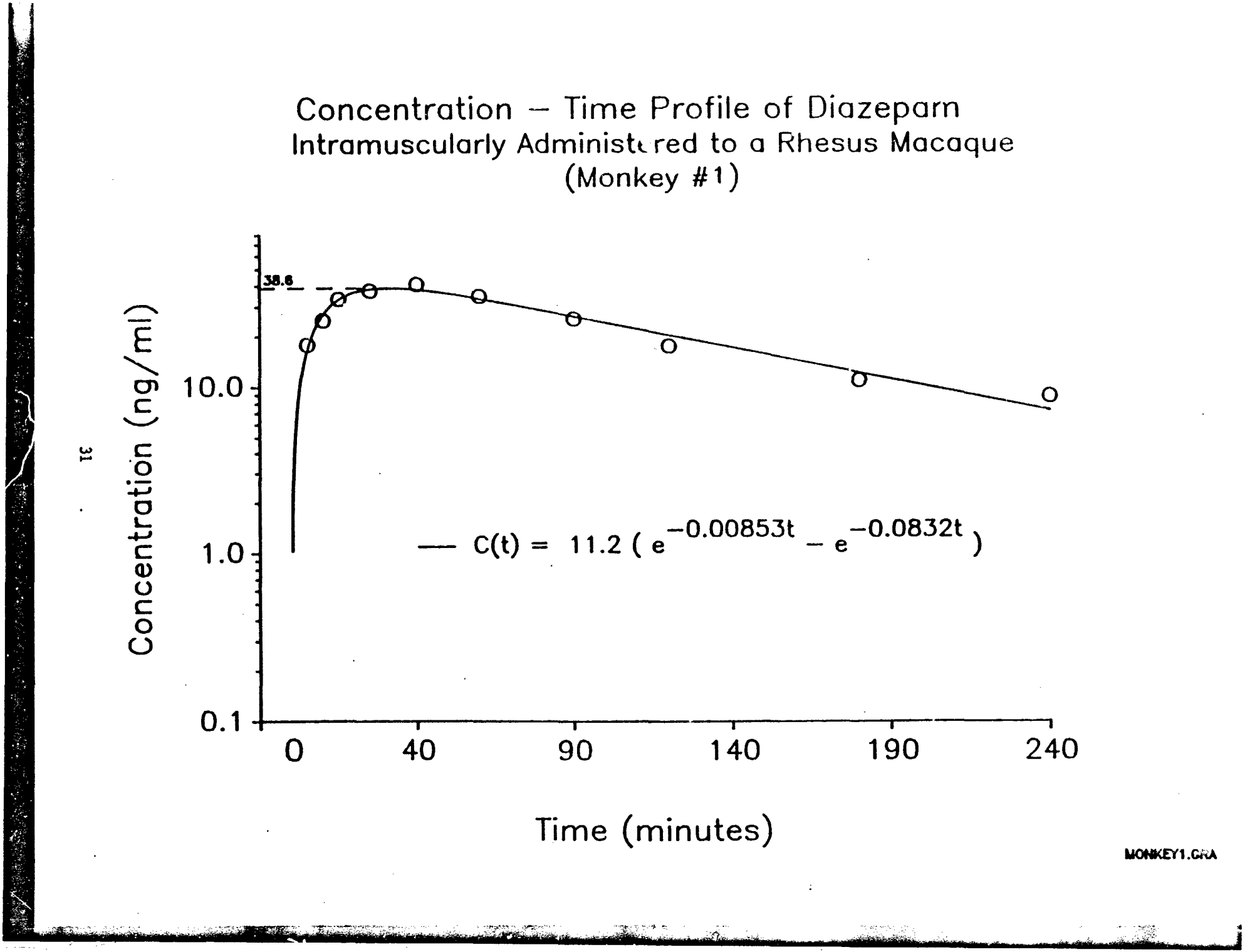
Sample No.	Expected Diazepam Conc. (ng/ml)	Detected Diazepam Conc. (ng/ml)	% Bias	Conc. Corrected for Zero	% Bias
4915	0	22.50	0.00	1.77	0.00
9177	0	21.30	0.00	1.07	0.00
8492	0	17.90	0.00	-2.33	0.00
Mean		20.73	0.00	-0.00	0.00
St. Dev.		2.48	0.00	2.48	0.00
3909	6.25	29.40	-370.40	8.67	-38.67
1714	6.25	27.20	-335.20	6.47	-3.47
0625	6.25	27.20	-335.20	6.47	-3.47
Mean		27.93	-346.93	7.20	-15.20
St. Dev.		1.27	16.59	1.27	16.59
1038	12.5	32.10	-156.80	11.37	9.07
8381	12.5	31.60	-152.80	10.87	13.07
4381	12.5	34.60	-176.80	13.87	-10.93
Mean		32.77	-162.13	12.03	3.73
St. Dev.		1.61	10.50	1.61	10.50
9527	25	46.40	-85.60	25.67	-2.67
4848	25	55.80	-123.20	35.07	-40.27
3192	25	45.30	-81.20	24.57	1.73
Mean		49.17	-96.67	28.43	-13.73
St. Dev.		5.77	18.85	5.77	18.85
6634	50	77.00	-54.00	56.27	-12.53
0907	50	73.10	-46.20	52.37	-4.73
0752	50	73.90	-47.80	53.17	-6.33
Mean		74.67	-49.33	53.93	-7.87
St. Dev.		2.06	3.36	2.06	3.36
7317	100	118.00	-18.00	97.27	2.73
2839	100	116.70	-16.70	95.97	4.03
3242	100	125.40	-25.40	104.67	-4.67
Mean		120.03	-20.03	99.30	0.70
St. Dev.		4.69	3.83	4.69	3.83
8524	200	201.90	-0.95	181.17	9.42
8895	200	204.10	-2.05	183.37	8.32
1634	200	208.10	-4.05	187.37	6.32
Mean		204.70	-2.35	183.97	8.02
St. Dev.		3.14	1.28	3.14	1.28

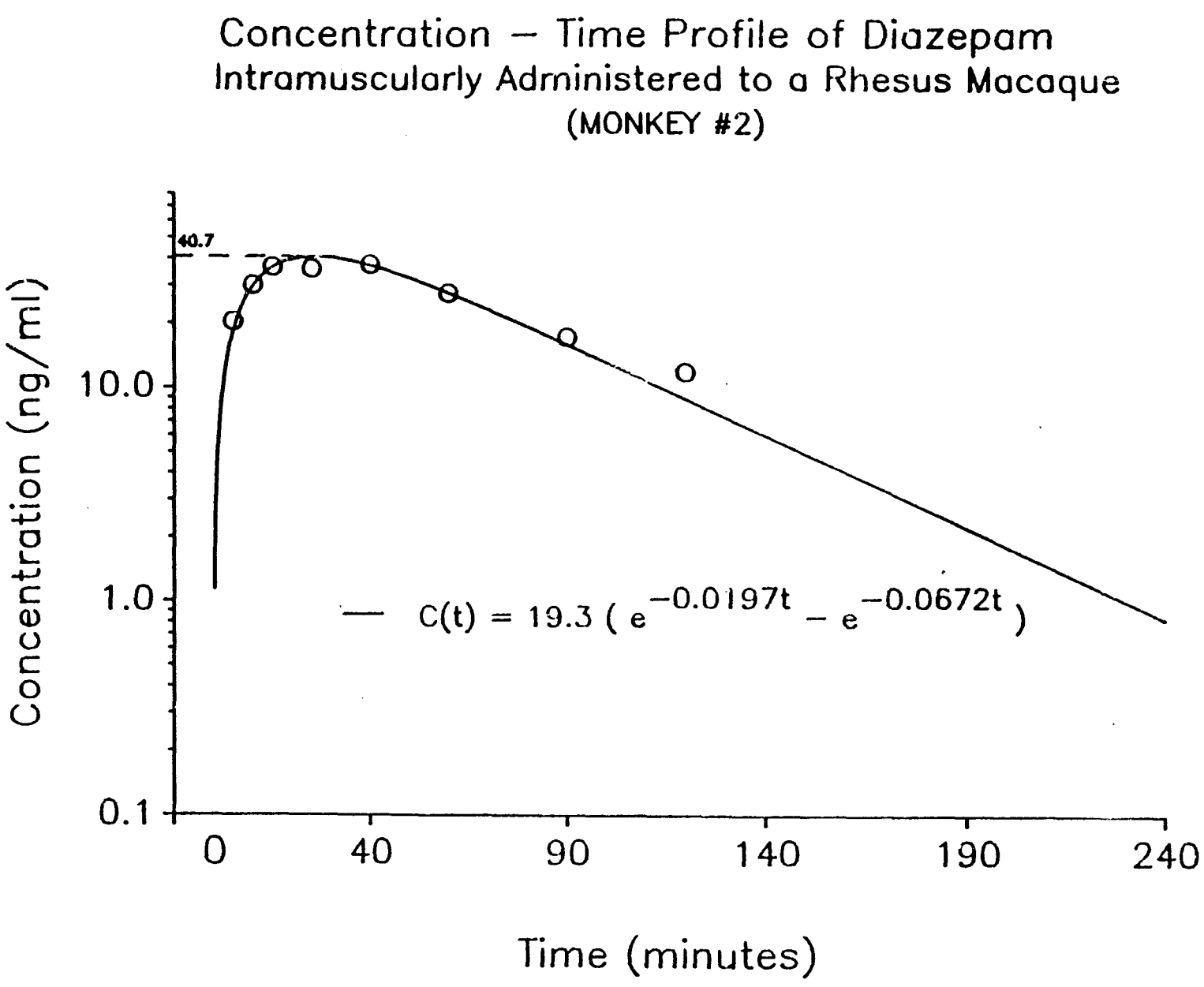
QUALITY CONTROL FOR DIAZEPAM MONKEY STUDY
(Diazepam Expiration Date: July 1988)

Sample No.	Expected Diazepam Conc. (ng/ml)	Detected Diazepam Conc. (ng/ml)	% Bias	Conc. Corrected for Zero	% Bias
2345	100.00	111.00	-11.00	90.27	9.73
2482	100.00	113.10	-13.10	92.37	7.63
1965	100.00	115.60	-15.60	94.87	5.13
Mean		113.23	-13.23	92.50	7.50
St. Dev.		1.88	1.88	1.88	1.88
8542	12.50	37.30	-198.40	16.57	-32.53
1927	12.50	34.60	-176.80	13.87	-10.93
0998	12.50	39.60	-216.80	18.87	-50.93
Mean		37.17	-197.33	16.43	-31.47
St. Dev.		2.04	16.35	2.04	16.35

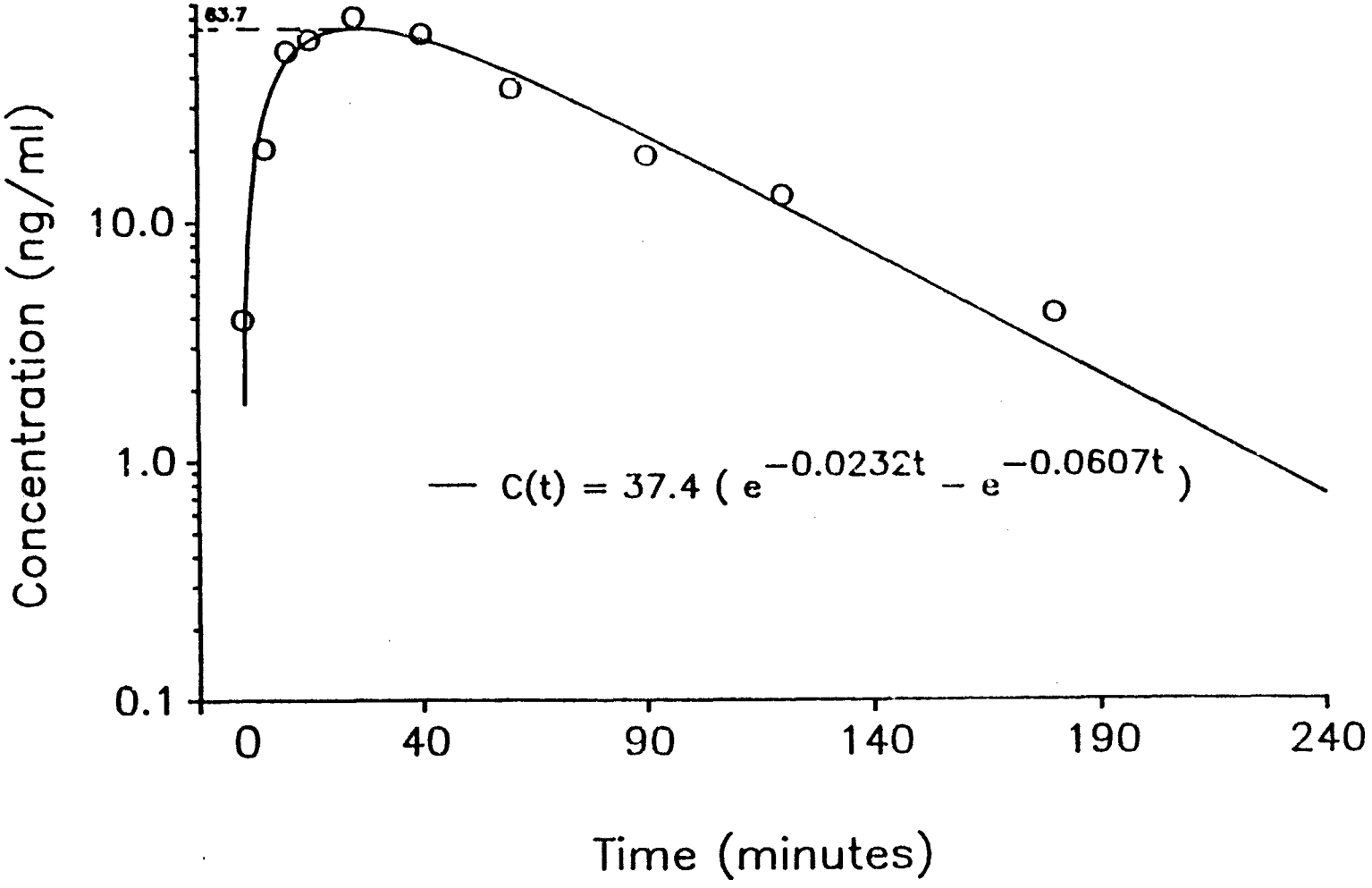
APPENDIX C

DIAZEPAM CONCENTRATION-TIME PROFILES

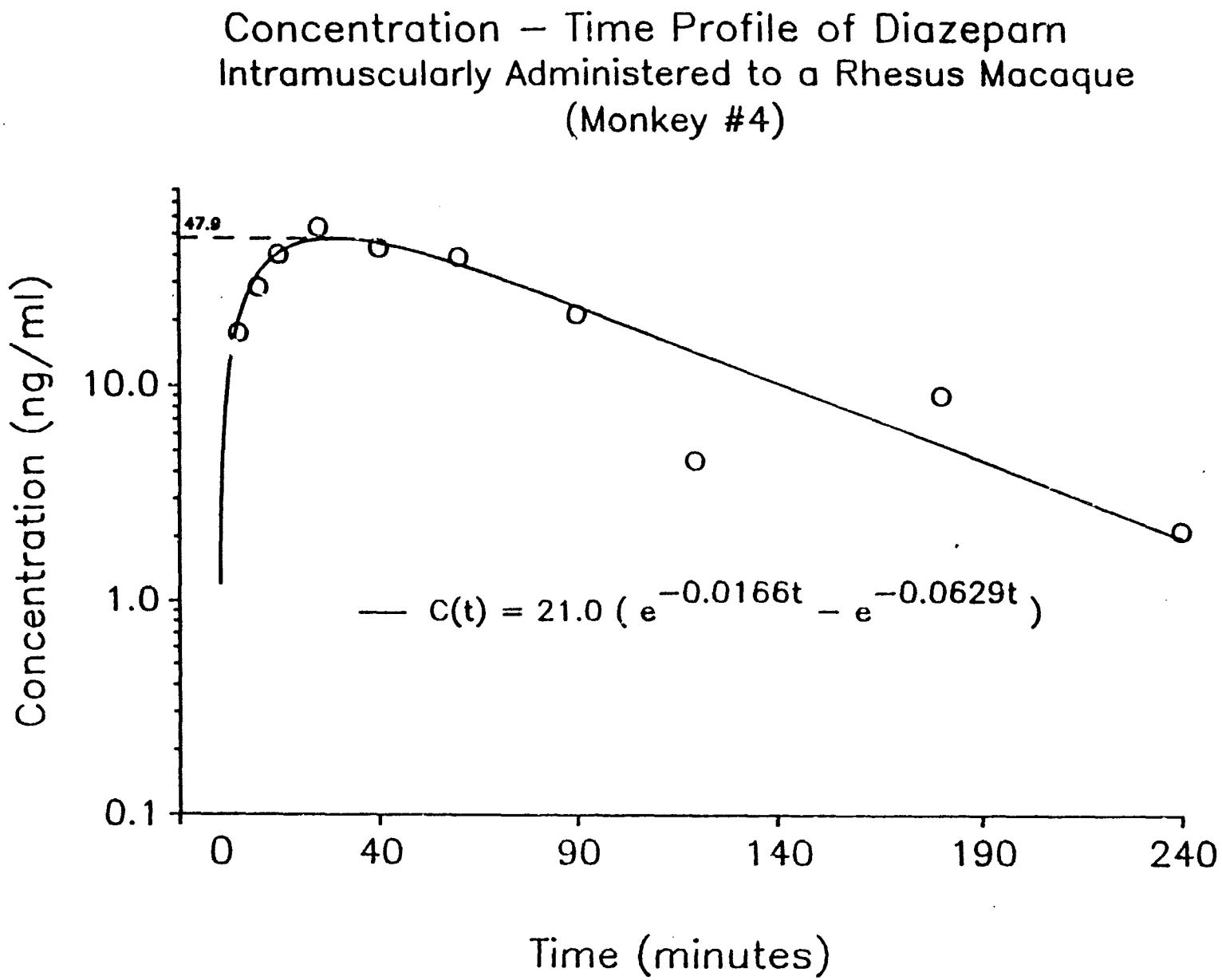

Concentration – Time Profile of Diazepam
Intramuscularly Administered to a Rhesus Macaque
(Monkey #1)
38.6
10.0
1.0
0.1
Concentration (ng/ml)
C(t) = 11.2 (e^−0.00853t − e^−0.0832t)
0
40
90
140
190
240
Time (minutes)
MONKEY1.GRA


Concentration – Time Profile of Diazepam
Intramuscularly Administered to a Rhesus Macaque
(MONKEY #2)
40.7
10.0
1.0
0.1
Concentration (ng/ml)
C(t) = 19.3 (e^−0.0197t − e^−0.0672t)
0
40
90
140
190
240
Time (minutes)

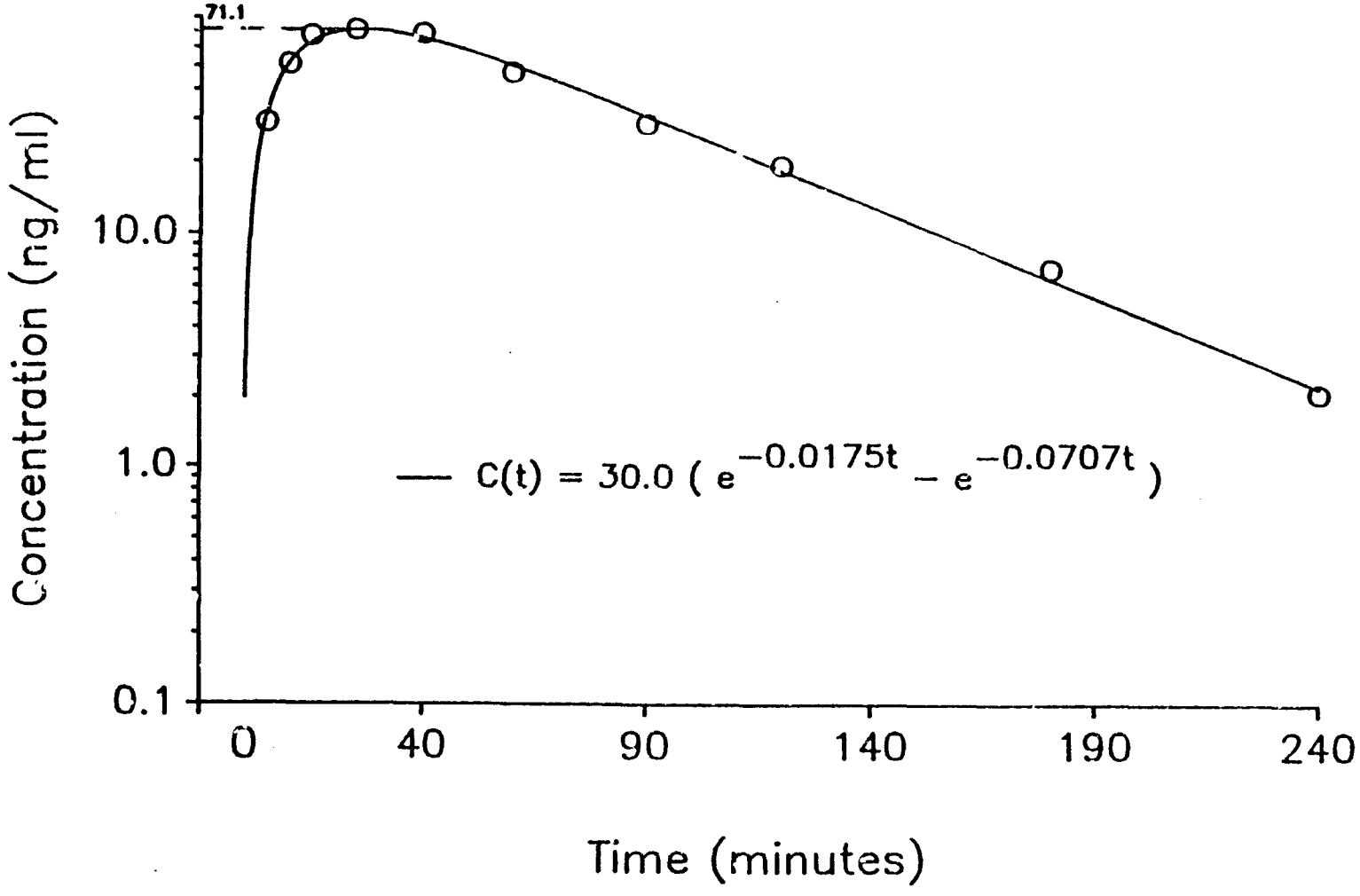
Concentration – Time Profile of Diazepam
Intramuscularly Administered to a Rhesus Macaque
(Monkey #3)

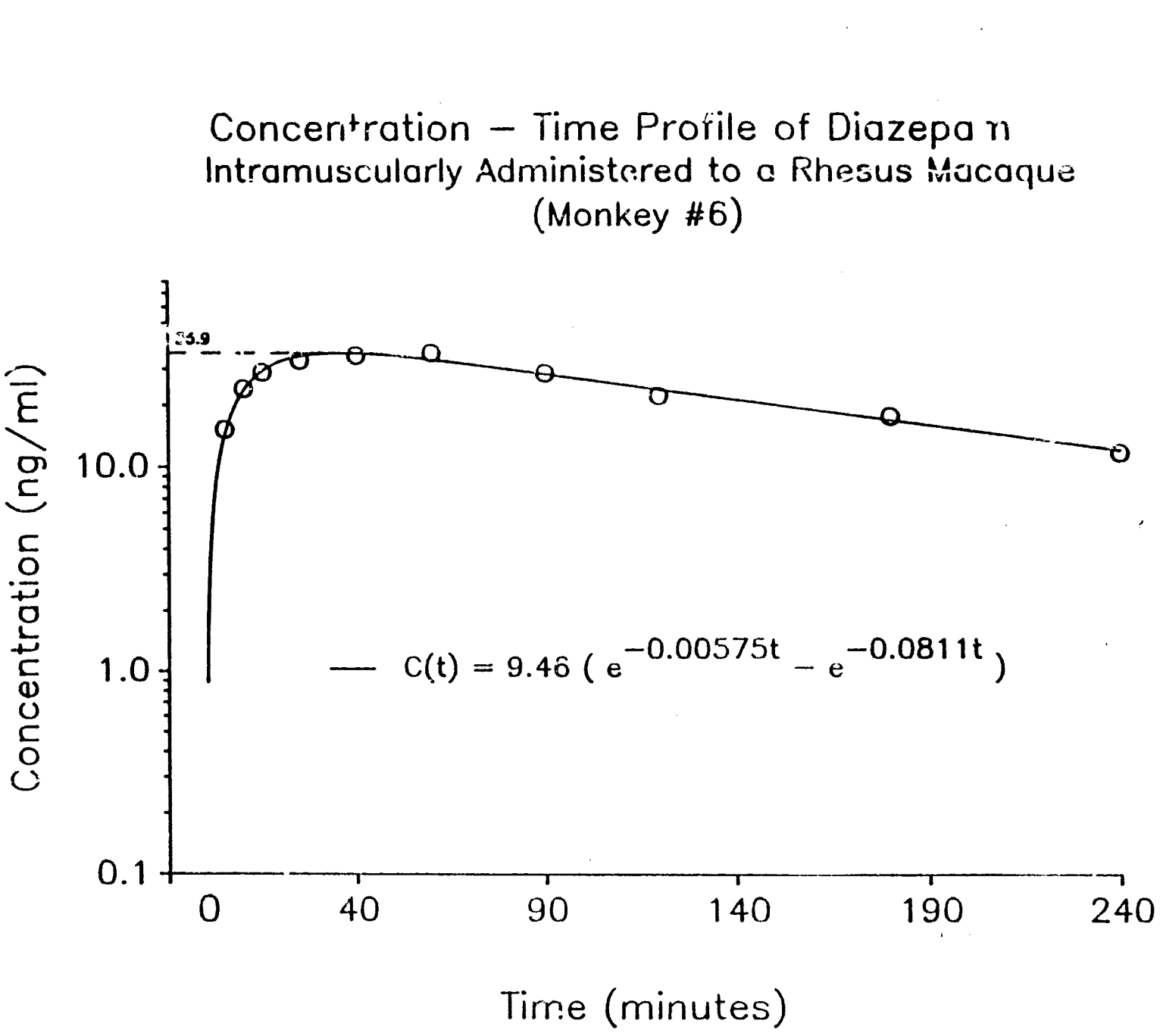


MONKEY3.GRA


Concentration – Time Profile of Diazepam
Intramuscularly Administered to a Rhesus Macaque
(Monkey #4)
47.9
Concentration (ng/ml)
10.0
1.0
0.1
C(t) = 21.0 (e^−0.0166t − e^−0.0629t)
0
40
90
140
190
240
Time (minutes)

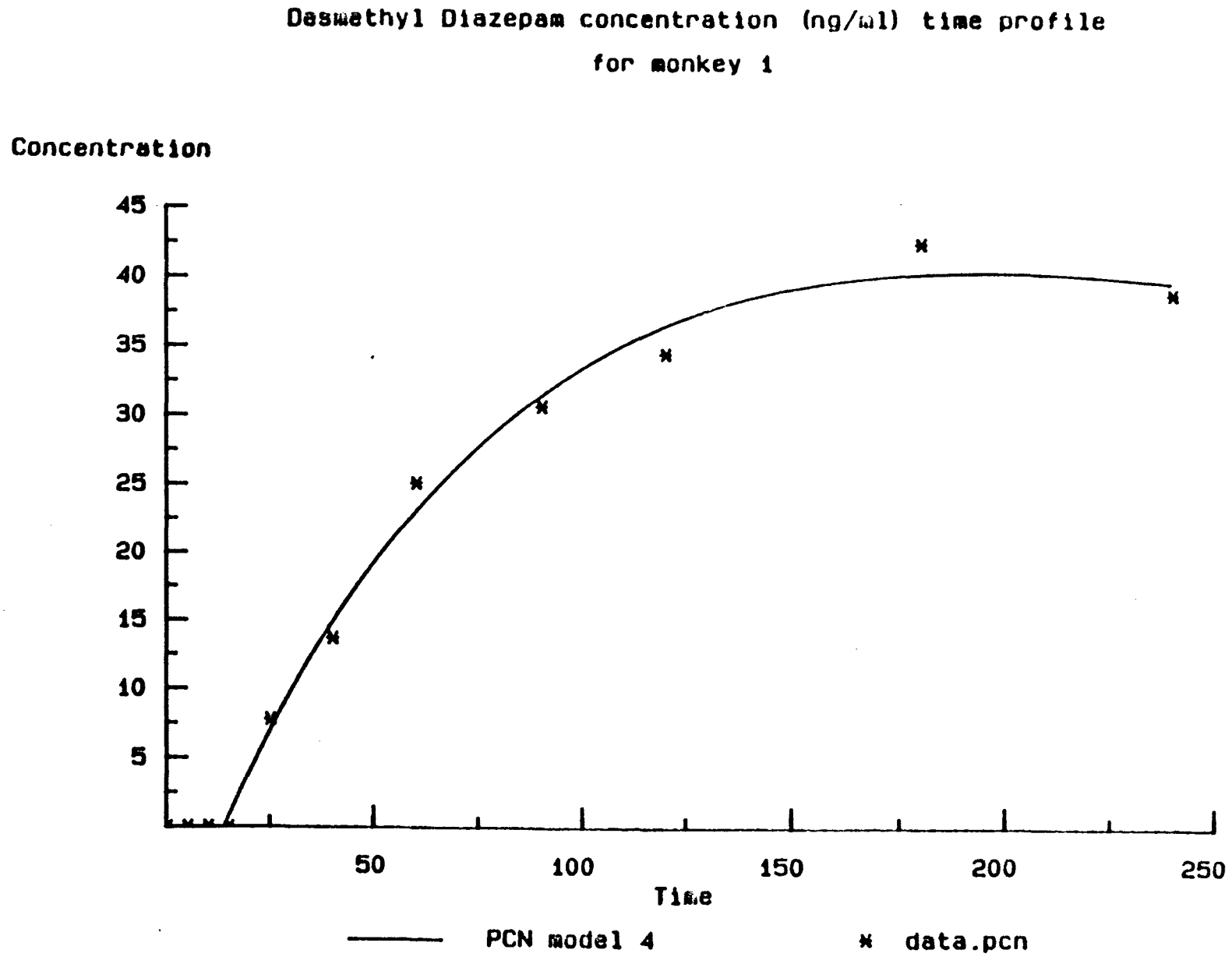
Concentration – Time Profile of Diazepam
Intramuscularly Administered to a Rhesus Macaque
(Monkey #5)



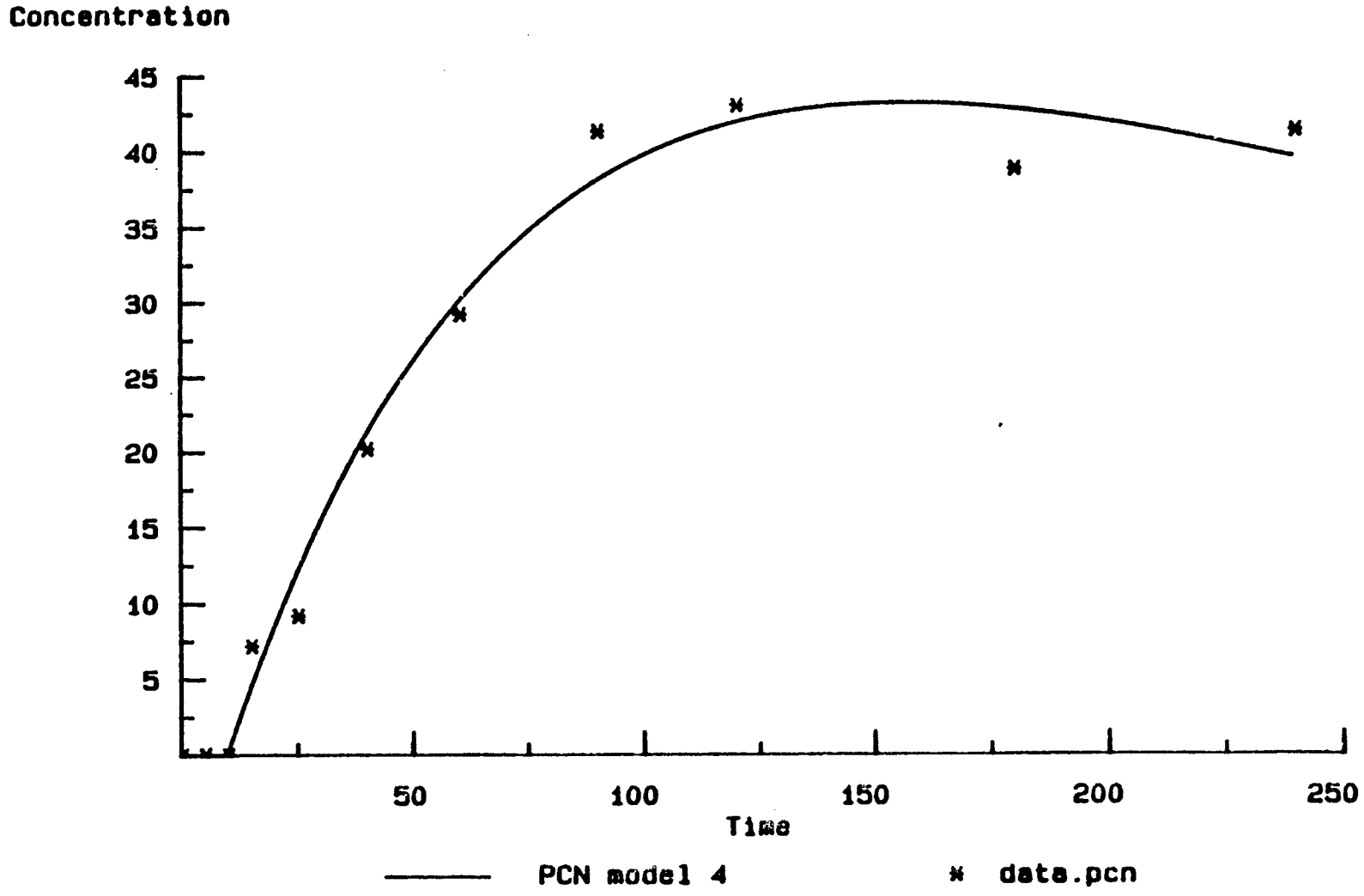

Concentration – Time Profile of Diazepam
Intramuscularly Administered to a Rhesus Macaque
(Monkey #6)
Concentration (ng/ml)
10.0
1.0
0.1
0
40
90
140
190
240
Time (minutes)
C(t) = 9.46 (e^−0.00575t − e^−0.0811t)

APPENDIX D

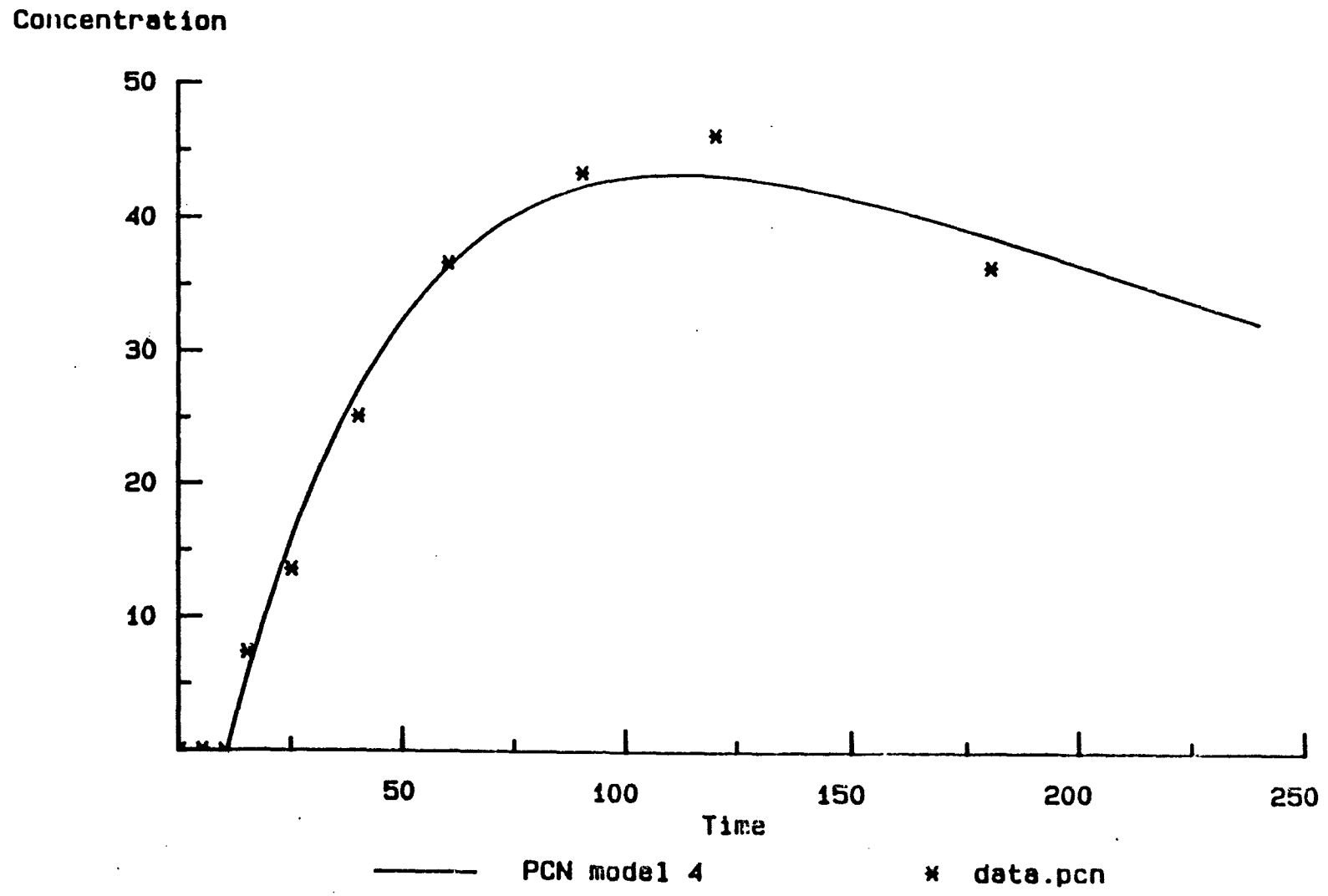
DESMETHYL DIAZEPAM CONCENTRATION-TIME PROFILES

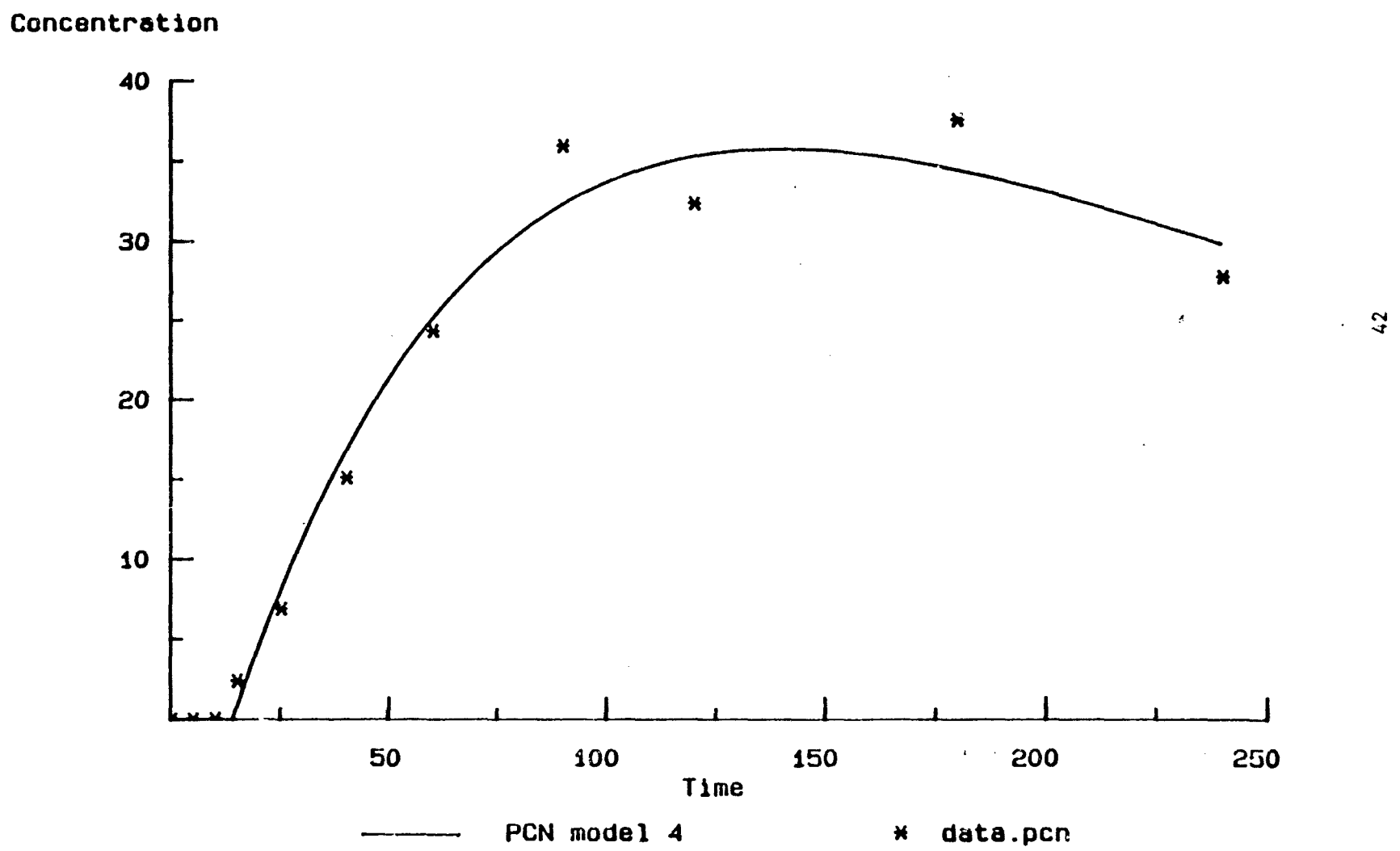

Desmethyl Diazepam concentration (ng/ml) time profile
for monkey 1
Concentration
45
40
35
30
25
20
15
10
5
50
100
150
200
250
Time
PCN model 4
data.pcn

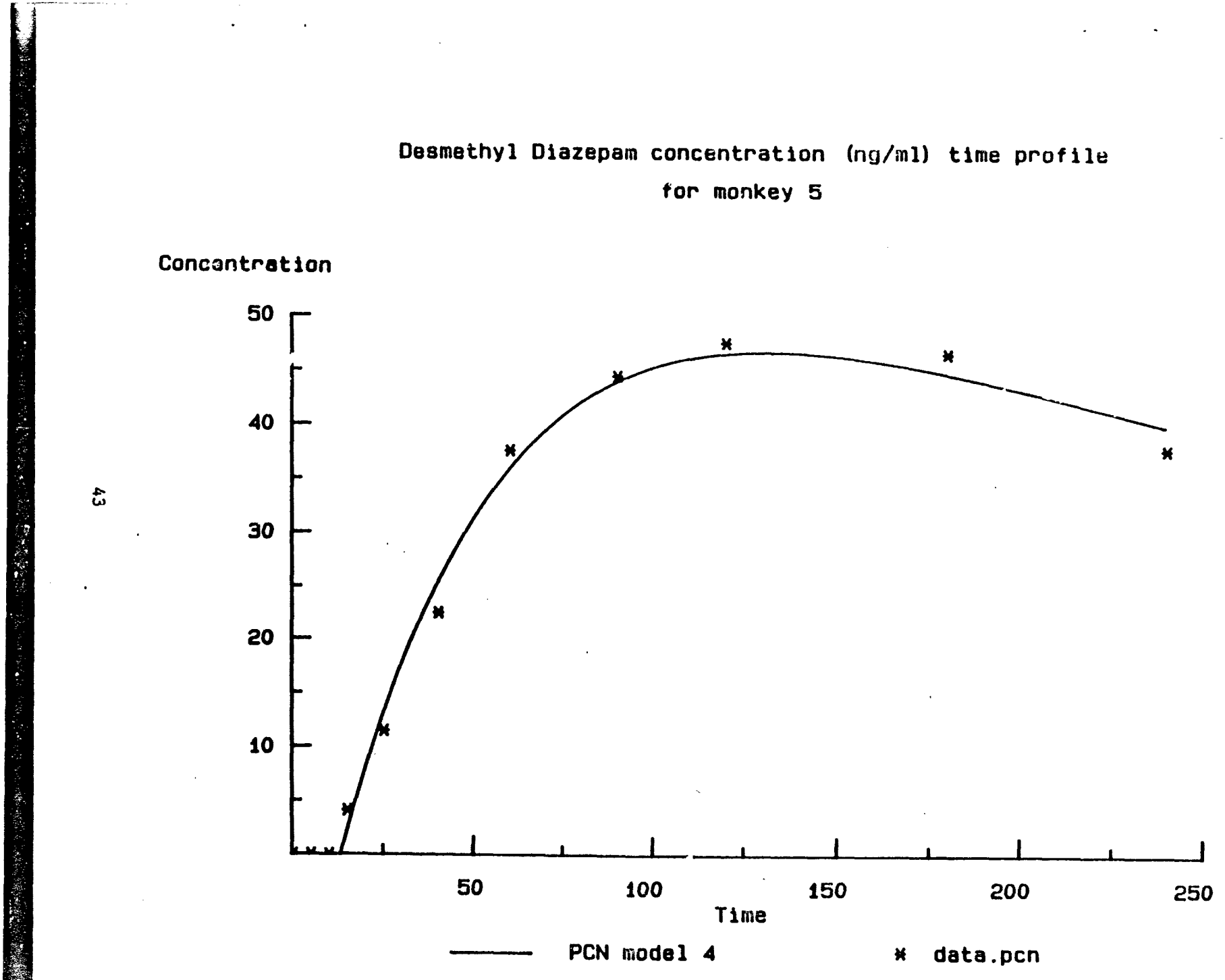
Desmethyl Diazepam concentration (ng/ml) time profile
for monkey 2

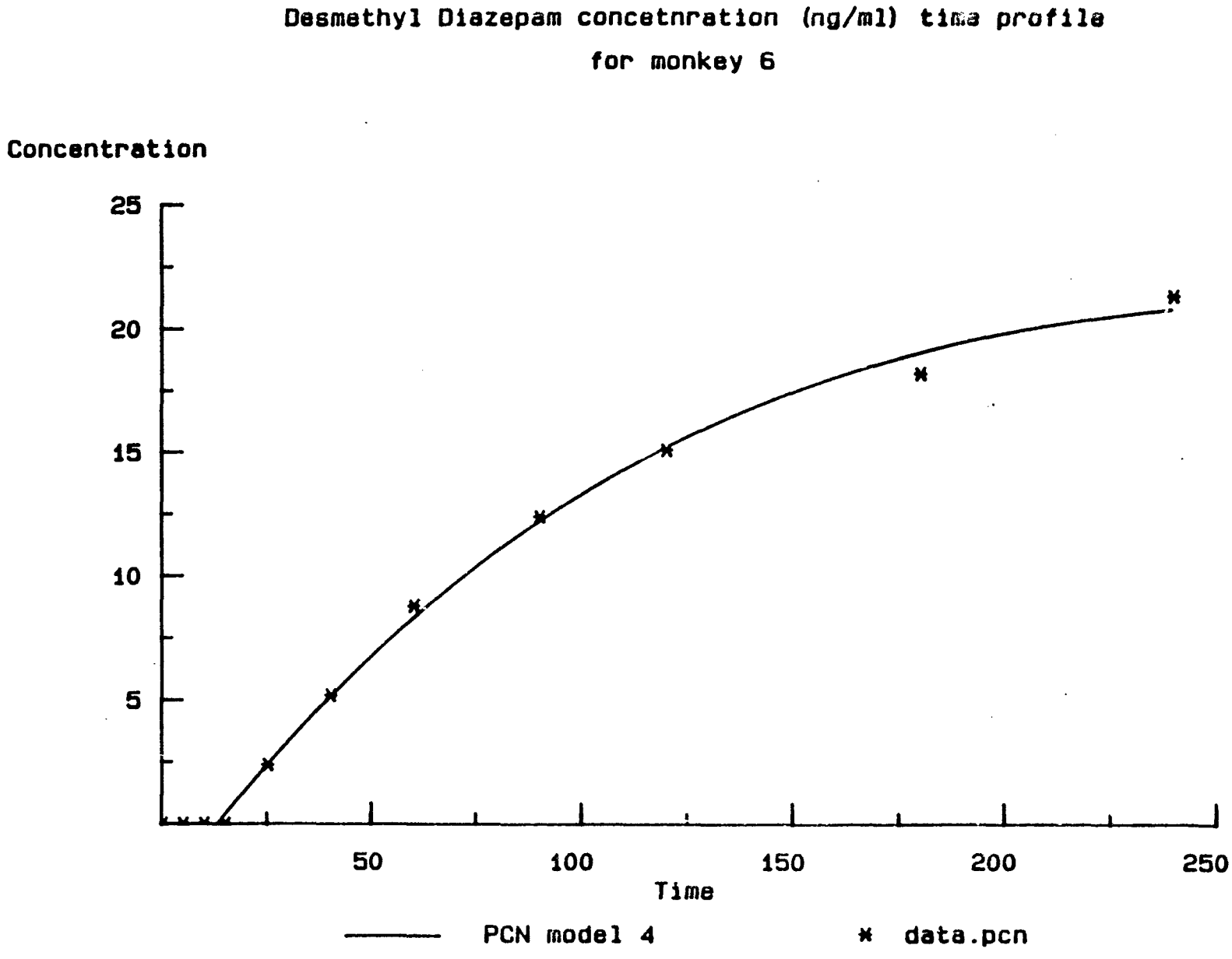


Desmethyl Diazepam concentration (ng/ml) time profile
for monkey 3




Desmethyl Diazepam concentration (ng/ml) time Profile
for monkey 4
Concentration
40
30
20
10
50
100
150
200
250
Time
PCN model 4
data.pcn


Desmethyl Diazepam concentration (ng/ml) time profile
for monkey 5
Concentration
50
40
30
20
10
50
100
150
200
250
Time
PCN model 4
data.pcn


Desmethyl Diazepam concetnration (ng/ml) time profile
for monkey 6
Concentration
25
20
15
10
5
50
100
150
200
250
Time
PCN model 4
data.pcn

APPENDIX E

PHARMACOKINETIC ESTIMATES OF DIAZEPAM AND DESMETHYLDIAZEPAM FOR EACH ANIMAL

Pharmacokinetic parameters (S.D.) of diazepam intramuscularly administered to rhesus monkeys.

		ANIMAL NUMBER 1	2	3	4	5	6	Mean	S.D.*
Vd	L/kg	1.98	1.47	0.87	1.30	0.89	2.28	1.47	0.53
	S.D.**	0.17	0.25	0.15	0.28	0.05	0.08	0.15	
k01	1/min	0.083	0.067	0.061	0.063	0.071	0.081	0.071	0.009
	S.D.**	0.010	0.018	0.016	0.022	0.006	0.006	0.013	
k10	1/min	0.009	0.020	0.023	0.017	0.018	0.006	0.015	0.006
	S.D.**	0.001	0.003	0.004	0.004	0.001	0.000	0.002	
AUC	ng*min/ml	5874	3415	4975	4648	6416	7638	5494	1348
	S.D.**	272	256	335	472	171	302	301	
Cl	ml/min/kg	17.0	29.3	20.1	21.5	15.6	13.1	19.4	5.2
	S.D.**	0.8	2.2	1.4	2.2	0.4	0.5	1.2	
t1/2 of k01	(min)	8.3	10.3	11.4	11.0	9.8	8.6	9.9	1.2
	S.D.**	1.0	2.8	3.0	3.9	0.9	0.7	2.0	
t1/2 of k10	(min)	81.3	34.9	29.9	41.7	39.5	120.5	58.0	32.6
	S.D.**	6.8	6.0	5.4	9.5	2.3	7.8	6.3	
Tmax	min	30.5	25.8	25.6	28.8	26.2	35.1	28.7	3.4
	S.D.**	2.0	2.9	2.5	4.3	1.1	1.5	2.4	
Cmax	ng/ml	38.6	40.7	63.7	47.9	71.1	35.9	49.6	13.2
	S.D.**	1.3	2.7	3.8	4.2	1.7	0.8	2.4	
sum of sq. res		28.9	45.9	227.0	192.7	51.1	14.9	93.4	83.7
r of y,yhat		0.991	0.990	0.983	0.974	0.997	0.994	47.202	75.115

* S.D. of pharmacokinetic estimates for all animals.

** S.D. of the pharmacokinetic estimates for each animal determined from the nonlinear regression computer program, PCNONLIN.

Pharmacokinetic parameters (S.D.) of desmethyldiazepam intramuscularly administered to rhesus monkey

	Animal #	1	2	3	4	5	6	Mean	S.D.*
D/Vd	ng/ml	59.0	62.5	62.5	62.5	62.5	62.1	61.8	1.3
	S.D.**	135.6	161.3	204.9	93.1	410.3	5.0	26.1	
Tlag	(min)	14.0	9.9	10.8	14.1	13.3	13.3	12.6	1.6
	S.D.**	1.9	2.1	2.0	2.6	1.4	1.8	2.0	
k01	(1/min)	0.011	0.014	0.021	0.013	0.020	0.003	0.014	0.006
	S.D.**	0.006	0.008	0.009	0.011	0.005	0.042	0.013	
k10	(1/min)	0.002	0.003	0.004	0.004	0.002	0.004	0.003	0.001
	S.D.**	0.003	0.003	0.003	0.005	0.001	0.044	0.010	
AUC	(ng*min/ml)	27993	24579	17202.5	14019	25131	15636	20760	5328
	S.D.**	21687	15121	7881.5	6235	7308	18847	12847	
t1/2 of k01	(min)	60.4	47.9	33.1	53.6	34.1	203.9	72.2	59.7
	S.D.**	32.9	25.0	14.7	44.4	8.4	2490.8	436.0	
t1/2 of k10	(min)	328.6	272.6	190.8	155.5	278.7	174.7	233.5	63.2
	S.D.**	396.5	271.9	144.7	172.5	122.4	1941.1	508.2	
Tmax	(min)	195.0	155.6	111.9	139.8	131.0	285.2	169.8	57.6
	S.D.**	21.9	12.5	7.0	9.5	6.1	71.1	21.3	
Cmax	(ng/ml)	40.3	43.2	43.3	35.7	46.6	21.1	38.4	8.4
	S.D.**	1.0	1.8	1.5	1.7	1.3	1.1	1.4	
sum of sq. res		17.1	47.7	28.0	43.1	26.2	1.4	27.2	15.5
r of y,yhat		0.997	0.993	0.996	0.991	0.997	0.999	1.00	17.1

* S.D. of pharmacokinetic estimates for all animals.

** S.D. of the pharmacokinetic estimates for each animal determined from the nonlinear regression computer program, PCNONLIN.

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